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CD14 Expression in Injured Patients Correlates with Outcome

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Objective

The authors determined the correlation between monocyte CD14 expression and outcome in severely injured patients.

Summary Background Data

Human leukocyte antigen-DR (HLA-DR) expression CD14 positive monocytes correlates with the development of major infection and subsequent death in severely injured patients. Recent studies show that CD14 is not only a marker for mature monocytes, but also is an important endotoxin/lipopolysaccharide receptor.

Methods

Flow cytometry data obtained by dual staining techniques (CD14 and HLA-DR) of monocytes in 213 severely injured patients were analyzed over a 30-day period. Outcome criteria included survival and the development of both major and minor infections.

Results

The percentage of cells expressing CD14 (%CD14) correlated with clinical outcome, reaching significance ($p < 0.05$) between noninfected survivors ($n = 74$) and nonsurvivors ($n = 21$) at days 3, 7, 11, 17, 24, and 30. At days 3, 7, and 17, the %CD14 also was different between noninfected and infected survivors. After 7 days, differences were only seen between survivors and nonsurvivors ($p < 0.05$). The mean fluorescence intensity (MC CD14) in monocytes of all patients was significantly reduced at day 3 compared with day 1 and remained low for 30 days ($p < 0.05$). The nonsurvivor group had consistently low MC CD14 values, which were significant at day 5 ($p < 0.05$).

Conclusions

In addition to HLA-DR expression, CD14 expression on monocytes is an indicator of clinical outcome after injury and could represent a more precise target for treatment.

Infection continues to be a major problem for trauma patients,[1,2] despite improvements in field rescue, transportation, resuscitation, definitive surgery, intensive care, and appropriate antibiotic use. Various scoring systems were developed to determine trauma impact and to predict outcome,[3–6] but none predicted the risk of infection. Subsequently, it was shown that the expression of human leukocyte antigen-DR (HLA-DR) on monocytes correlated with the development of major infection and could be used to predict infection and assess clinical outcome after injury.[7,8] In 1992, a prospective, randomized, double-blind, multicenter trial was performed with 213

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trauma patients who were at high risk for infection.[9] These patients received either placebo or recombinant interferon-gamma (rIFN-γ). Dual staining techniques and flow cytometry were used to measure monocyte HLA-DR expression. Mature monocytes were identified with the monoclonal anti-CD14 antibody Mo2. The study confirmed that HLA-DR expression on CD14-positive monocytes correlated with the occurrence of infection and outcome after trauma. Treatment with rIFN-γ did not affect final outcome, although the incidence of severe infections was reduced. A second multicenter trial of rIFN-γ in severely injured patients has just been reported.[10] Not unlike the first trial,[9] the second study also came to confounding conclusions as to clinical efficacy and provided no new laboratory observations to clarify the issue of intended immune reconstitution.

Interferon-gamma is a potent immunomodulator with well-documented effects on the monocyte/macrophage defense systems. Recombinant IFN-γ enhances hydrogen peroxide generation[11] and Fc receptor expression[12–14] and also increases the expression of HLA class I[15] and HLA class II antigens on monocytes.[16–19] Because of this wide spectrum of activities, rIFN-γ, was introduced as an immunomodulator in cancer patients in 1985.[20]

Recent studies have shown that CD14 is not only a marker for mature monocytes, but it also is an important endotoxin/lipopolysaccharide (LPS) receptor.[21] CD14 is a 55-kd protein found as a glycosylphosphatidyl inositol-linked protein on the membrane surface of mononuclear phagocytes and as a soluble protein in the blood. There currently is ample evidence that LPS bound to LPS-binding protein (LBP) interacts with the CD14 receptor.[22] Lipopolysaccharide is an important component of the outer membrane of all gram-negative bacteria and is a well-recognized component responsible for many of the toxic manifestations of severe gram-negative sepsis.[23]

The goal of this study was to assess monocyte CD14 expression and its relationship to outcome in severely injured patients and to further clarify the original work previously presented in this journal.[8]

PATIENTS AND METHODS

Two hundred thirteen patients were enrolled in a randomized, prospective, double-blind clinical trial at four major trauma centers (University of Louisville School of Medicine, Louisville, KY; University of Medicine and Dentistry of New Jersey, Newark, NJ; State University of New York at Buffalo, Buffalo, NY; University of Michigan, Ann Arbor, MI), as described previously.[9] Entry criteria included patients who were older than 15 years of age whose major injury was manifested by an Injury Severity Score of 20 or greater, and who also had a documented presence of bacterial contamination as a result of the injury. Patients not admitted to the study were those with major burns, renal insufficiency (creatinine level > 3.0 mg/mL), hepatic insufficiency (direct bilirubin concentration > 3.0 mg/mL), and those who where pregnant or lactating. Patients requiring ongoing therapy with known immunosuppressive agents, such as corticosteroids, zidovudine, or cytotoxic chemotherapy agents, also were excluded from the study. Recombinant IFN-γ1b (100 μg; Genentech, Inc., San Francisco, CA) or placebo was administered each day for 10 consecutive days subcutaneously or until discharge from the hospital. Of the 213 randomized patients, 12 were excluded because they did not meet study eligibility requirements, as were 8 who died of closed head injury within the first 3 days. Cultures were obtained when infections occurred, and appropriate sensitivities were determined. The outcome criteria included survival and the development of both major and minor infections, according to clinical criteria, as appropriately confirmed by cultures.

Monocyte HLA-DR expression and CD14 expression were measured by dual monoclonal antibody staining and flow cytometry over a 30-day period. Forty microliters of whole blood (less than 1×10^6 leukocytes) were mixed with 5 μL (2.5 μg) of fluorescein isothiocyanate-coupled Mo2 monoclonal antibody (FITC-Mo2, Coulter Immunology, Hialeah, FL) and with 20 μL (0.25 μg) of phycoerythrin-coupled antihuman HLA-DR (Becton-Dickinson, Rutherford, NJ). The mixture was incubated at 4 C for 25 minutes. After the erythrocytes were removed by hypotonic lysis, the mixture was washed once with fluorescent treponemal antibody-hemagglutination buffer (BBL Microbiology Systems, Cockeysville, MD) and fixed on an Ortho Cytofluorograph IIs flow cytometer (Orthodiagnostics, Westwood, CA) configured for simultaneous two-color (red and green) fluorescence analysis. Results were described as *percentage of cells* expressing CD14 (%CD14), or *mean flourescence intensity* for CD14 (MC CD14) or HLA-DR (MC HLA-DR). The monocyte gate was set according to routine position for monocytes in the sideways-scatter and the forward scatter for mononuclear cells.[24]

Differential leukocyte counts were performed for each patient and assessed as absolute number and percentage of monocytes, lymphocytes, and polymorphonuclear leukocytes.

Statistical analysis was performed using chi-square analysis to compare the correlation between rIFN-γ treatment and final outcome and analysis of variance to compare the data from the noninfected survivors, the infected survivors, and the nonsurvivors, most of whom died of the effects of infection.

RESULTS

Of the eligible 193 patients, 97 were treated with rIFN-γ and 96 patients received placebo. Infections occurred in

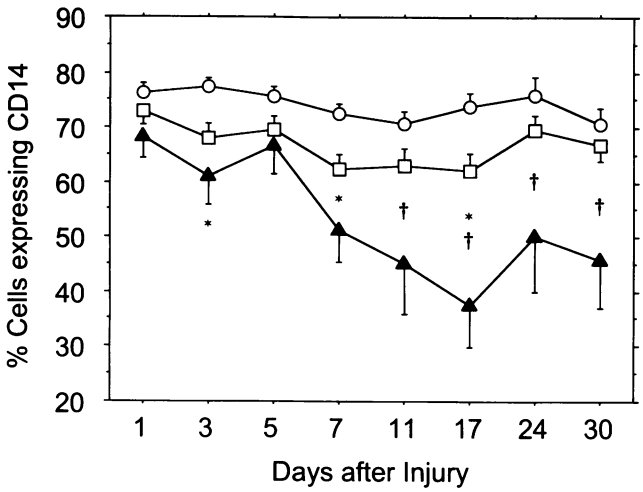

Figure 1. Correlation of patient outcome with percentage of cells expressing CD14. ○ = noninfected survivors; □ = infected survivors; ▲ = nonsurvivors. *p < 0.05 comparing noninfected survivors with infected survivors and with nonsurvivors, †p < 0.05 comparing survivors with nonsurvivors. Values are mean ± standard error.

119 patients, and 21 patients died of infection. Figure 1 shows the continuous down-regulation of the percentage of monocytes expressing CD14 in nonsurvivors compared with survivors from both the placebo and rIFN-γ groups. There was a significant difference ($p < 0.05$) in %CD14 expression between noninfected survivors and nonsurvivors at days 3, 7, 11, 17, 24, and 30. Although %CD14 was not significant at day 5, our results clearly show that CD14 expression was significantly down-regulated at day 5, based on MC CD14. At days 3, 7, and 17, the %CD14 also was different between noninfected and infected survivors; however, after 7 days, significant differences were seen only between survivors and nonsurvivors.

Three days after injury, MC CD14 (Fig. 2) was significantly reduced in all groups, compared with day 1, and remained low ($p < 0.05$) throughout the observation period. Nonsurvivors had consistently lower MC CD14 values compared with the noninfected or infected survivors. The only significant difference in MC CD14 among the three groups occurred at day 5.

Treatment with rIFN-γ for 10 days caused a significant up-regulation in MC HLA-DR expression (Fig. 3A), which occurred in all patients treated with rIFN-γ, irrespective of clinical outcome, and was followed by a return to placebo levels. Mean fluorescence intensity in CD14 showed persistently lower values in the rIFN-γ group when compared with the placebo group (Fig. 3B); however, significant difference ($p < 0.05$) occurred only at day 5. There was no correlation between rIFN-γ treatment and clinical outcome.

A similar pattern of HLA-DR expression on CD14-positive monocytes occurred when compared with all cells analyzed from the monocytes cluster in the scatter cytogram (Fig. 4). Human leukocyte antigen-DR expression on all monocytes (Fig. 4A) was significantly different between the noninfected survivors and the nonsurvivors ($p < 0.05$) at days 3, 5, 7, 11, 17, 24, and 30. Between day 3 and day 11, it further differentiated infected survivors from noninfected survivors. The only difference from the values analyzing all monocytes (Fig. 4B) was found at day 17, when HLA-DR expression on CD14-positive monocytes also could distinguish infected survivors from noninfected survivors ($p < 0.05$).

The number of leukocytes was significantly increased in nonsurvivors when compared with noninfected survivors (days 8, 11, 17, 24, and 30), in infected survivors when compared with noninfected survivors (days 8, 11, 24, and 30) and in nonsurvivors when compared with infected survivors (days 17, 24, and 30) The differential blood count showed no difference in the absolute number of lymphocytes and a moderate but not significant decrease in absolute numbers of monocytes in nonsurvivors. However, the percentage of lymphocytes showed significant lower values for the noninfected survivors when compared with infected survivors or nonsurvivors (days 8, 11, 17, and 30). The percentage of monocytes showed consistent low values for nonsurvivors and significant differences when noninfected survivors were compared with nonsurvivors (days 5, 8, and 17) or when noninfected survivors were compared with infected survivors (days 5, 8, 17, and 24). These results reflect the relative polymorphonuclear leukocyte increase in infected survivors and nonsurvivors after trauma.

DISCUSSION

Our findings are consistent with others[25,26] in associating a down-regulation of cells expressing CD14 in septic

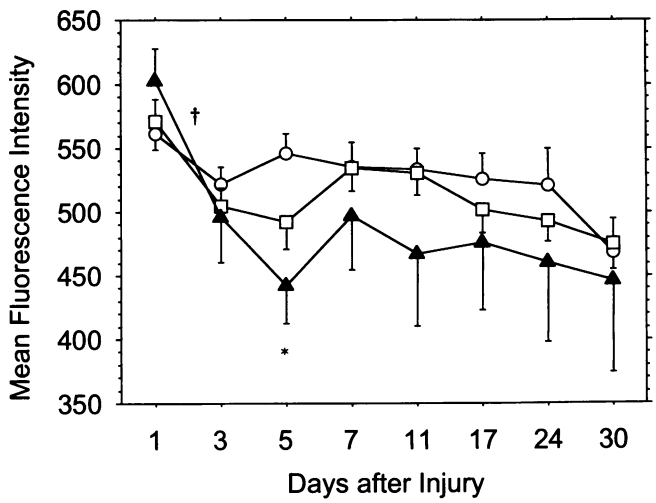

Figure 2. Correlation of patient outcome with densitiy of CD14, expressed as mean fluorescence intensity. ○ = noninfected survivors; □ = infected survivors; ▲ = nonsurvivors. *p < 0.05 comparing noninfected survivors with infected survivors and with nonsurvivors, †p < 0.05 between the mean value of all groups at day 1 compared to all following days. Values are mean ± standard error.

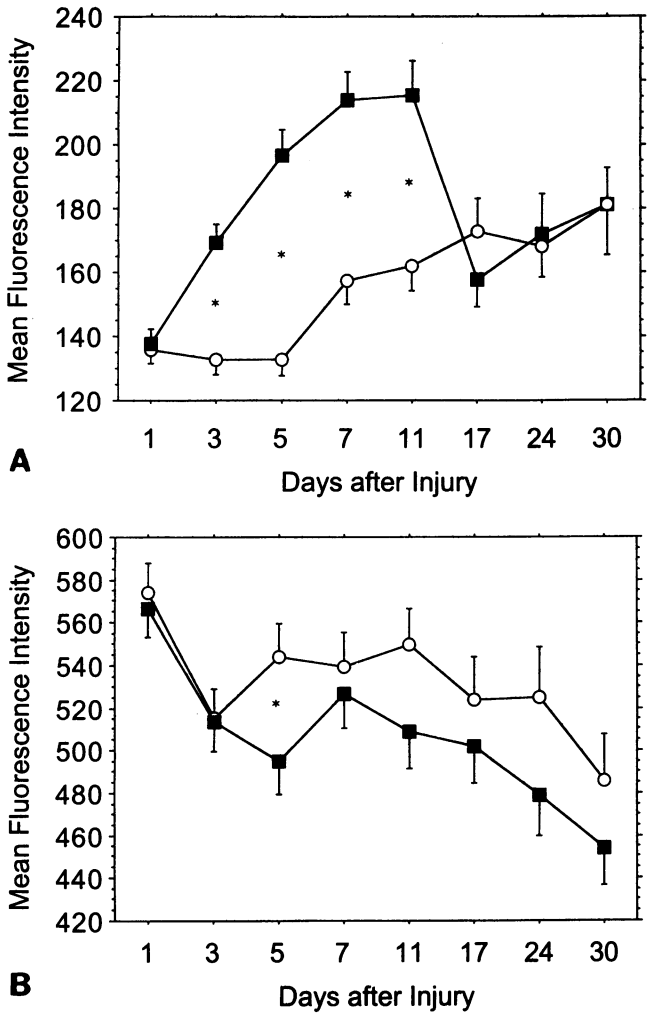

Figure 3. Influence of recombinant interferon-gamma (rIFN-γ) on human leukocyte antigen-DR expression (A) and CD14 expression (B) on human monocytes in injured patients. ■ = rIFN-γ group; O = placebo group. *p < 0.05. Values are mean ± standard error.

patients. However, the correlation between down-regulation of CD14 expression and death from infection has not been shown previously. Moreover, many studies analyzed soluble CD14, but very few investigated membrane-bound CD14.

To date, there are at least four known LPS receptors:[27] 1) CD14, 2) a 73-kd LBP on murine splenocytes, 3) CD18 (involved in the nonopsonic recognition of LPS), and 4) an acetyl LDL receptor on monocytes/macrophages (involved in the uptake and detoxification of LPS/lipid A). CD14 clearly seems to have a major role in LPS-mediated biological actions, such as release of tumor necrosis factor-alpha, interleukin-1, or interleukin-6.[28] Recent studies argue for a multichain LPS receptor including CD14, a receptor with common signal transducing subunits deriving its specificity from unique ligand-binding subunits.[22] An often mentioned putative LPS receptor, a 70-kd protein on murine lymphocytes and macrophages, has been identified as cell-bound albumin rather than a functional receptor for LPS.[29] The 70-kd protein also is capable of binding peptidoglycan, lipoteichoic acid, heparin, and sulfated heparinoids. Our data show a clear and significant reduction of CD14 receptor density after day 3 compared with day 1 (Fig. 2). These data suggest a shedding of CD14 as described by Bazil and Strominger.[30]

Soluble CD14 in serum has been reported in concentrations of 2 to 6 μg/mL in healthy volunteers[21]; however, in polytraumatized patients, soluble CD14 levels decreased to mean values of 1.7 μg/mL immediately after injury.[31] Six days post-trauma, soluble CD14 levels were elevated to mean values of 4.9 μg (compared with

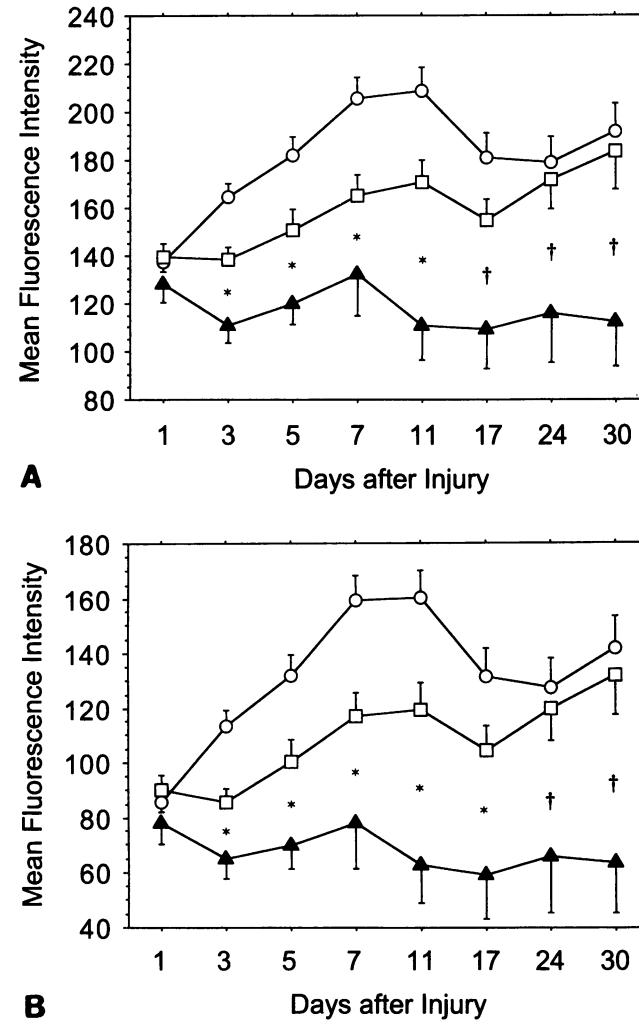

Figure 4. Correlation of patient outcome and density of human leukocyte antigen-DR antigen expression on all monocytes gated for flow cytometry (A) and on monocytes expressing CD14 (B). O = noninfected survivors; □ = infected survivors; ▲ = nonsurvivors. *p < 0.05 comparing noninfected survivors with infected survivors and with nonsurvivors. †p < 0.05 comparing noninfected survivors with nonsurvivors. Values are mean ± standard error.

their normal value of 3.7 μg/mL), and they remained elevated for another 8 days. Patients with the most severe injuries (Injury Severity Score > 45) continued to have high soluble CD14 levels after 14 days, whereas patients with an Injury Severity Score of less than 45 showed a return to normal levels. Unfortunately, Kruger et al.[31] did not report the final outcome of these patients.

Soluble CD14 has been suggested as a a target for a therapeutic approach in sepsis based on the capacity of soluble CD14 to bind LPS-LBP complexes.[32] It has been postulated that elevated soluble CD14 serum levels in polytraumatized patients may indicate a physiologic protective mechanism against excessive monocyte mediator production.[33] However, Wright[34] noted that humans can respond fatally to LPS in the presence of a molar excess of soluble CD14. During sepsis, peak concentrations of LPS may reach 2×10^{-11} mol/L, whereas soluble CD14 is measured at concentrations of 10^{-7} mol/L. The question is whether soluble CD14 is secreted (or shed from the cell surface) to bind LPS-LBP complexes or if soluble CD14 is shed from the surface to reduce the responding elements to the LPS-LBP complex on the cell surface. A continuous decrease of membrane-bound CD14 expression, as seen in the patients with either minor or severe infections, might reflect a reduced response threshold of monocytes to produce mediators. This continuous decrease, together with a lack in HLA-DR up-regulation, may be fatal. These effects are thought to be protective immediately after injury, but could result in reduced host defense several days after injury, when the patient is especially vulnerable to infection.

The results of early HLA-DR increase in patients without infection, the delayed increase of patients with minor infections, and the essential lack of HLA-DR up-regulation in patients who died of infections have been reported previously.[8] In our study, the pattern of HLA-DR expression on CD14-positive cells showed no difference from the HLA-DR expression on all cells analyzed from the monocytes cluster in the scatter cytogram. This result demonstrates that 1) HLA-DR expression on all monocytes correlates with the occurrence of infection and outcome after injury and 2) monocytes do not have to be specified as presumably mature monocytes by CD14-specific monoclonal antibody like Mo2. Similar results on the modulation of HLA-DR expression after IFN-γ treatment are supported by other *in vitro* and *in vivo* studies.[16–19]

The problem of the accuracy of the traditional "monocyte gate" and the possibility of confounding results due to changes in absolute cell numbers require discussion. Some mononuclear cells, like natural killer cells or lymphoblasts, can be "trapped" in the monocyte gate.[24] Therefore, a stronger lymphocyte response would reduce the percentage of (CD14-positive) monocytes in the traditional monocyte gate. Conversely, our results show an identical number of absolute lymphocytes and a significant decrease in the percentage of lymphocytes in patients with complicated outcome. Therefore, the reduction of CD14 is *not* due to an increased lymphocyte response. Mean channel fluorescence measurements can only be used as an indicator of cell-surface antigen expression when the cell size remains constant. We did not perform controls to ensure that the size of the CD14-positive cells did not change, but we gated all cells for our analysis in the traditional (monocyte) area defined by the forward and sideways scatter. This scattergram reflects leukocytes by their cell size, their internal structure or granularity, and their cellular content, such as DNA and RNA.[24]

Our results show that monocyte CD14 expression is an independent factor that correlates with clinical outcome after injury. The results for CD14 expression in studies from other laboratories are less clear and occasionally contradictory. Membrane-bound CD14 down-regulation after incubation with rIFN-γ was reported by Landmann et al.,[18] but their results were obtained *in vitro,* and a subsequent *in vivo* study showed increased CD14 expression during rIFN-γ therapy in cancer patients.[35]

Human leukocyte antigen-DR expression on all monocytes correlates with the occurrence of infection and outcome after injury, irrespective of CD14 expression. Moreover, we showed that CD14 expression on monocytes is an independent factor correlated with outcome after injury. We conclude that CD14 expression on monocytes is another indicator of clinical outcome after injury and that the use of CD14 as a potential target for therapeutic interventions seems reasonable.

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